# Influence of Endogenous and Exogenous Electron Donors and Trichloroethylene Oxidation Toxicity on Trichloroethylene Oxidation by Methanotrophic Cultures from a Groundwater Aquifer

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Trichloroethylene (TCE)-transforming aquifer methanotrophs were evaluated for the influence of TCE oxidation toxicity and the effect of reductant availability on TCE transformation rates during methane starvation. TCE oxidation at relatively low (6 mg liter$^{-1}$) TCE concentrations significantly reduced subsequent methane utilization in mixed and pure cultures tested and reduced the number of viable cells in the pure culture *Methylomonas* sp. strain MM2 by an order of magnitude. Perchloroethylene, tested at the same concentration, had no effect on the cultures. Neither the TCE itself nor the aqueous intermediates were responsible for the toxic effect, and it is suggested that TCE oxidation toxicity may have resulted from reactive intermediates that attacked cellular macromolecules. During starvation, all methanotrophs tested exhibited a decline in TCE transformation rates, and this decline followed exponential decay. Formate, provided as an exogenous electron donor, increased TCE transformation rates in *Methylomonas* sp. strain MM2, but not in mixed culture MM1 or unidentified isolate, CSC-1. Mixed culture MM2 did not transform TCE after 15 h of starvation, but mixed cultures MM1 and MM3 did. The methanotrophs in mixed cultures MM1 and MM3, and the unidentified isolate CSC-1 that was isolated from mixed culture MM1 contained lipid inclusions, whereas the methanotrophs of mixed culture MM2 and *Methylomonas* sp. strain MM2 did not. It is proposed that lipid storage granules serve as an endogenous source of electrons for TCE oxidation during methane starvation.

Trichloroethylene (TCE), a widely used organic solvent and degreasing agent, belongs to the group of most frequently detected groundwater contaminants (44). TCE is a suspected carcinogen (24). Furthermore, it has been demonstrated to undergo reductive dechlorination in anaerobic environments, with vinyl chloride, a known mammalian carcinogen and mutagen (22, 33), as one of the most significant products (7, 31, 39, 40). Wilson and Wilson (46) were the first to indicate that TCE might be susceptible to microbial transformation under aerobic conditions, which would not result in formation of toxic or carcinogenic volatile chlorinated products. Since then, numerous researchers have addressed this phenomenon and discovered various microorganisms to be capable of transforming TCE. In all cases examined so far, the active microorganisms contain catabolic oxygenases with relaxed substrate specificities which catalyze a fortuitous oxygenation of TCE upon being stimulated with their respective substrates (43). Such microorganisms include methanotrophs (1, 9, 13, 14, 16, 21, 30, 37), propane oxidizers (41), ethylene oxidizers (13), toluene, phenol, or cresol oxidizers (10, 12, 26, 27, 28, 42), 2,4-dichlorophenoxyacetic acid oxidizers (12), and ammonia oxidizers (2, 38).

In developing process applications for treatment of TCE contamination that rely on transformation by catabolic oxygenases, two issues that must be evaluated are toxicity and reductant supply. Elevated TCE concentrations (in the range of 50 mg liter$^{-1}$) have been shown to be toxic to methanotrophs, inhibiting TCE transformation (13, 30) and methane utilization (13). Janssen and colleagues (18), working with mixed methanotrophic cultures, observed that 6 mg of TCE liter$^{-1}$ inhibited methane-dependent growth but not methanol-dependent growth. From this, they suggested that reactive intermediates may cause toxic effects (18). Wackett and Gibson (42) observed toxicity during TCE oxidation by *Pseudomonas putida* F1 and suggested that toxic intermediates were responsible. Wackett and Householder (43) subsequently demonstrated that the TCE had to be metabolically activated by the toluene dioxygenase of *P. putida* F1 to produce toxic effects. In methanotrophic treatment applications, even at low TCE concentrations, TCE oxidation itself may be toxic to the TCE-oxidizing cells and may result in reduction of the active biomass.

As with other catabolic oxygenases, the methane monooxygenase enzyme system (MMO) of methanotrophs requires electrons (reducing power) to carry out its catabolic function. During growth on methane, reducing power is regenerated by the mineralization of methane to carbon dioxide (17). During the oxidation of a cometabolite such as TCE, however, the reductant supply is not regenerated. Methane can be provided as an electron donor, but methane is also a competitive inhibitor of TCE transformation (13, 19, 19b, 30, 34). Transformation by resting cells in the absence of methane does occur (1, 13, 14, 19, 30, 34, 37), but during extended methane starvation, when no substrate is available to regenerate the reductant, cells can be depleted of reducing power. Formate, an electron donor but not a growth substrate (17), has been shown to stimulate resting-cell transformation of TCE in a pure culture (30) and during in situ biodegradation of chlorinated ethenes in a groundwater aquifer (34).

Whereas formate may serve as an exogenous source of electrons during methane starvation, storage polymers such as lipid inclusions may serve as an endogenous source of reductant in aquifer microorganisms. Methanotrophs are

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known to accumulate both lipid and polyglucose inclusions (3, 20). The presence of endogenous reserves may have great significance in an environment such as the subsurface, where nutrients and carbon and energy sources are likely to be scarce. Poindexter (32) suggested that lipid inclusions should be expected in microorganisms adapted to oligotrophic environments, and Ghiorse and Balkwill (11) found lipid storage granules in numerous subsurface bacteria. No studies have been reported evaluating TCE transformation by storage polymer-containing methanotrophs.

A field study evaluating methane-stimulated in situ biodegradation of chlorinated ethenes was conducted at a site located at the Moffett Naval Air Station in California (34, 35). In conjunction with this field study we have enriched and isolated TCE-transforming methanotrophic mixed and pure cultures from the Moffet Field groundwater aquifer (13, 14). An objective of our work is to evaluate the response of these microorganisms to conditions that may be considered operational parameters for in situ bioremediation or waste treatment applications. We previously described the effect that mineral medium formulation had on TCE transformation by two different cultures: a mixed culture containing type II methanotrophs and a pure culture possessing type I intracytoplasmic membranes, *Methylomonas* sp. strain MM2 (14). In this paper we report the toxic effect of TCE oxidation and the responses of the various cultures, including lipid granule-containing methanotrophs, to methane starvation and formate amendment.

(Preliminary reports of this study have been published [12a, 13a, 14a].)

## MATERIALS AND METHODS

**Organisms and culture conditions.** Three different mixed cultures, MM1, MM2, and MM3, were enriched from a groundwater aquifer located in Moffett Field Naval Air Station, Mountain View, Calif., with methane as carbon source, as described elsewhere (13, 14). *Methylomonas* sp. strain MM2 (13, 14) was isolated previously in our laboratory. A second unidentified methane-utilizing isolate, CSC-1, was isolated from the mixed culture MM1, following the same methods as described for the isolation of *Methylomonas* sp. strain MM2 (13, 14). The cultures were grown in either modified Fogel mineral medium (9) or Whittenbury medium (45) to mid-exponential growth phase (biomass density, 0.5 to 0.7 g [dry weight] liter$^{-1}$) in continuously stirred reactors under a continuous stream of 30 to 35% methane in air at room temperature (21 to 23°C). *Methylomonas* sp. strain MM2 and isolate CSC-1 were tested for heterotrophic contamination by streaking on one-fourth-strength tryptone-glucose plates (6 g of tryptone-glucose extract agar and 11.25 g of Noble agar liter$^{-1}$; Difco Laboratories, Detroit, Mich.).

**Microscopy.** The cultures were examined by light microscopy and by scanning and transmission electron microscopy as described previously (13, 14). Lipid inclusions were identified with transmission electron microscopy as well as with light microscopy by differential staining with Sudan black B (29).

**Analytical procedures.** Degradation experiments with $^{14}$C-labeled TCE, conducted to confirm that TCE was being biologically transformed and to determine the percentage of TCE carbon in the cell, carbon dioxide, and aqueous intermediate fractions, have been described elsewhere (13, 14). For TCE transformation and methane utilization studies, cultures were removed from the reactors, diluted with min-

eral medium, and transferred to bottles, which were incubated upside-down on a rotary shaker in a 21°C environmental chamber as described previously (13, 14). TCE transformation rates were determined by using unlabeled TCE (99+% spectrophotometric grade; Aldrich Chemical Co., Inc., Deerfield, Ill.) in the absence of methane or other growth substrate (resting-cell transformation). Formate, when provided as an exogenous electron donor for TCE transformation and an energy source for microorganisms, was added as sodium formate (Fisher Scientific Co., Fair Lawn, N.J.). Since 2 and 8 mM formate resulted in the same enhancement of TCE transformation rates, the lower concentration was used in further experiments. TCE transformation was monitored by measuring headspace concentrations by gas chromatography as described previously (13, 14). Methane, carbon dioxide, oxygen, and nitrogen were measured by monitoring headspace on a gas partitioner (14). Formate was measured by ion chromatography on a Dionex ionalyzer equipped with an ionpac AS4A separator column and a conductivity detector (Dionex Corp., Sunnyvale, Calif.), using 5 mM borate buffer. Cell biomass was determined on a dry weight basis, using 0.2-μm Supor filters (Gelman Sciences Inc., Ann Arbor, Mich.). CFU were enumerated by dilution plating on Noble agar plates made with Fogel mineral medium. (The same plating medium was used to maintain the cultures.) Monod kinetics for substrate degradation (25) were used to model the degradation of TCE and determine the pseudo-first-order rate coefficient, $k' = k/K_s$ (liter mg$^{-1}$ day$^{-1}$), as described previously (14). TCE transformation experiments described herein were performed at aqueous TCE concentrations ranging from 30 to 60 μg liter$^{-1}$.

**Electron donor availability during starvation experiments.** The effect of methane starvation on the ability of the mixed cultures to transform TCE was evaluated. The three mixed cultures, grown in Fogel medium, were incubated for 15 h in aerated stirred reactors without methane and then tested for TCE transformation and evaluated for lipid inclusions by transmission electron microscopy and staining with Sudan black B. One set of replicates was amended with methane at 0.3 mg liter$^{-1}$ when TCE was added, whereas another set of replicates did not receive methane. The TCE concentration used in this experiment was 60 μg liter$^{-1}$. Cell densities were 0.04 to 0.07 g (dry weight) liter$^{-1}$.

The effect of formate on TCE transformation was evaluated in *Methylomonas* sp. strain MM2, mixed culture MM1, and the unidentified isolate CSC-1, all grown in Whittenbury mineral medium. *Methylomonas* sp. strain MM2 was evaluated for zero-time TCE transformation rates, after which the culture in the reactor was split into two subcultures in 2-liter Erlenmeyer flasks. One subculture ("formate") was amended with 2 mM formate, which was measured by ion chromatography at 0 and 62 h. No formate was added to the other subculture ("none"). Both subcultures were stirred at room temperature (21 to 23°C) and periodically sampled over a 62-h period to be used in the experiments for evaluation of TCE oxidation rates. The formate-amended subculture received an additional 2 mM formate when it was diluted into the bottles for the transformation studies. The "none" subculture was also evaluated for formate addition at the time of TCE addition by adding 2 mM formate at the same time the TCE was added ("none + formate"). Mixed culture MM1 was similarly evaluated. Isolate CSC-1 was incubated in the stirred flask with no formate added and evaluated at the time of TCE addition with no formate ("none") and with 2 mM formate added ("none + formate"). The change in the

TCE transformation rates with time was evaluated. Exponential decay in the rate of TCE transformation with time was assumed, and a slope describing the reduction in rate with time was generated by plotting $[-\ln (rate_t/rate_0)]$ versus time. The TCE concentration used in these three experiments was 30 μg liter$^{-1}$. Cell densities were on the order of 0.08 g (dry weight) liter$^{-1}$ for *Methylomonas* sp. strain MM2, 0.02 g liter$^{-1}$ for mixed culture MM1, and 0.2 g liter$^{-1}$ for isolate CSC-1. CFU were enumerated at 62 h for the subcultures of *Methylomonas* sp. strain MM2.

**TCE oxidation toxicity experiments.** To evaluate TCE oxidation toxicity, the effects of TCE oxidation on subsequent methane utilization by mixed culture MM1 and *Methylomonas* sp. strain MM2 were evaluated. The cultures were grown in Whittenbury mineral medium. Replicate bottles of subcultures were amended with 2 mM formate and incubated with 6 mg of TCE liter$^{-1}$. In assay 1, *Methylomonas* sp. strain MM2 was also incubated with 6 mg of perchloroethylene (PCE) liter$^{-1}$, which has a similar chemical structure to TCE but is not transformed by methanotrophs. TCE and PCE were measured by gas chromatography as described before (13, 14). After approximately 2 h of incubation, the bottles were opened and the remaining TCE was removed by stripping with air. The bottles were then recapped and evaluated to ensure that all TCE and PCE had been removed, and methane was added. Bottles of subcultures which did not receive TCE were treated in the same way. In assay 2 with *Methylomonas* sp. strain MM2, the subcultures were centrifuged after incubation with TCE to separate the cells from the supernatant. The supernatants were then switched, so that the cells that had not oxidized TCE were evaluated for methane utilization in the culture medium that contained the aqueous TCE transformation intermediates, and the cells that had oxidized TCE were evaluated for methane utilization in culture medium that did not contain any TCE transformation intermediates. For assay 2, 1-ml samples were removed from each subculture before centrifugation and CFU were enumerated. Cell densities used in these experiments were 0.34 g (dry weight) liter$^{-1}$ for *Methylomonas* sp. strain MM2 assay 1, 0.52 g liter$^{-1}$ for *Methylomonas* sp. strain MM2 assay 2, and 0.28 g liter$^{-1}$ for mixed culture MM1.

## RESULTS

**Characterization of the cultures.** Mixed cultures MM1 and MM3 contained only methanotrophs with type II intracytoplasmic membrane structures (paired membranes inside the periphery of the cell [8]), whereas mixed culture MM2 contained methanotrophs with both type I (bundles of stacked membranes [8]) and type II membrane structures (13, 14). Unidentified methane-utilizing isolate CSC-1 contained type II membranes. Isolate CSC-1 exhibited constant, uniform colony and cell morphology with repeated single-colony isolation and, unlike the heterotrophs contained in mixed culture MM1, did not grow in the absence of methane on tryptone-glucose plates. Under the growth conditions of these experiments, methanotrophs in mixed cultures MM1 and MM3 and isolate CSC-1 possessed lipid inclusions, whereas *Methylomonas* sp. strain MM2 and the methanotrophs of mixed culture MM2 did not (Fig. 1).

**TCE transformation.** TCE transformation by these cultures at the low TCE concentrations used in the rate experiments (30 to 60 μg liter$^{-1}$) is adequately modeled with Monod kinetics (Fig. 2). Of the 77 psuedo-first-order rate coefficients ($k'$) generated in the experiments we report (see

Tables 1 and 2; Fig. 2 and 3), the data for 64 fit the kinetic model with correlation coefficients of 0.98 to 1.00, and the data for the remaining 13 fit the kinetic model with correlation coefficients of 0.89 to 0.97. *Methylomonas* sp. strain MM2, grown in Whittenbury medium and provided with 2 mM formate, has exhibited TCE transformation rates as great as 2.3 ± 0.05 liter mg$^{-1}$ day$^{-1}$ (1.6 ml mg$^{-1}$ min$^{-1}$) at 21°C (Fig. 2). Greatest rates observed for Whittenbury-grown mixed culture MM1 and isolate CSC-1 at 21°C were 0.64 ± 0.04 (0.45 ml mg$^{-1}$ min$^{-1}$) and 0.49 ± 0.01 (0.34 ml mg$^{-1}$ min$^{-1}$) liter mg$^{-1}$ day$^{-1}$, respectively.

**Electron donor availability during starvation.** Mixed cultures MM1, MM2, and MM3 grown in Fogel medium were aerobically incubated for 15 h without methane to evaluate the effects of starvation on TCE transformation. Mixed cultures MM1 and MM3, which contained methanotrophs that possessed lipid inclusions, continued to transform TCE and transformed TCE equally well whether or not amended with methane (Table 1). Mixed culture MM2 could not transform TCE without methane amendment. Moreover, when methane was added at the time of TCE transformation evaluation, the rates were much lower than for the other cultures (Table 1).

*Methylomonas* sp. strain MM2, mixed culture MM1, and isolate CSC-1, all grown in Whittenbury medium, were evaluated for the effect of formate amendment on TCE transformation during methane starvation. Formate amendment increased rates of transformation in *Methylomonas* sp. strain MM2 but not in mixed culture MM1 or isolate CSC-1 (Table 2; Fig. 3). The *Methylomonas* sp. strain MM2 subculture incubated with formate during methane starvation ("formate") exhibited enhanced rates of TCE transformation only during the first 10 h of starvation. However, when *Methylomonas* sp. strain MM2 was incubated without formate and formate was added at the same time the TCE was added ("none + formate"), the rates of transformation remained significantly enhanced throughout the entire 62 h of incubation (Fig. 3). At 0 h, the "formate" subcultures contained 2 mM formate and, at 62 h, 0.3 mM formate. CFU were enumerated at 62 h for *Methylomonas* sp. strain MM2 to determine whether the cultures were still viable. Subculture "none" contained 2 × 10$^7$ CFU ml$^{-1}$ and subculture "formate" contained 6 × 10$^7$ CFU ml$^{-1}$. The decrease in TCE transformation rates during starvation is presented in Table 2. As indicated by the correlation coefficients that describe the fit of the data to the model, the reduction of TCE transformation rates during starvation follows exponential decay (Table 2).

**TCE oxidation toxicity.** TCE oxidation impaired the ability of both mixed culture MM1 and *Methylomonas* sp. strain MM2 to utilize methane. Subcultures of both that had transformed TCE took three times longer to consume the methane present in the bottles than subcultures that were not exposed to TCE (Fig. 4). The mass of TCE oxidized relative to the cell mass was 0.028 for *Methylomonas* sp. strain MM2 assay 1, 0.017 for *Methylomonas* sp. strain MM2 assay 2, and 0.016 for mixed culture MM1. Incubation with PCE had no effect on subsequent methane utilization by *Methylomonas* sp. strain MM2 (Fig. 4a). To determine whether the aqueous intermediates resulting from TCE transformation had an inhibitory effect on methane utilization, the culture medium from *Methylomonas* sp. strain MM2 subculture that had oxidized TCE was switched with that from the subculture that had not been exposed to TCE (Fig. 4b). Culture medium containing TCE transformation intermediates had no effect on methane utilization. (As determined by ion chromatography and assays with $^{14}$C-labeled TCE, approx-
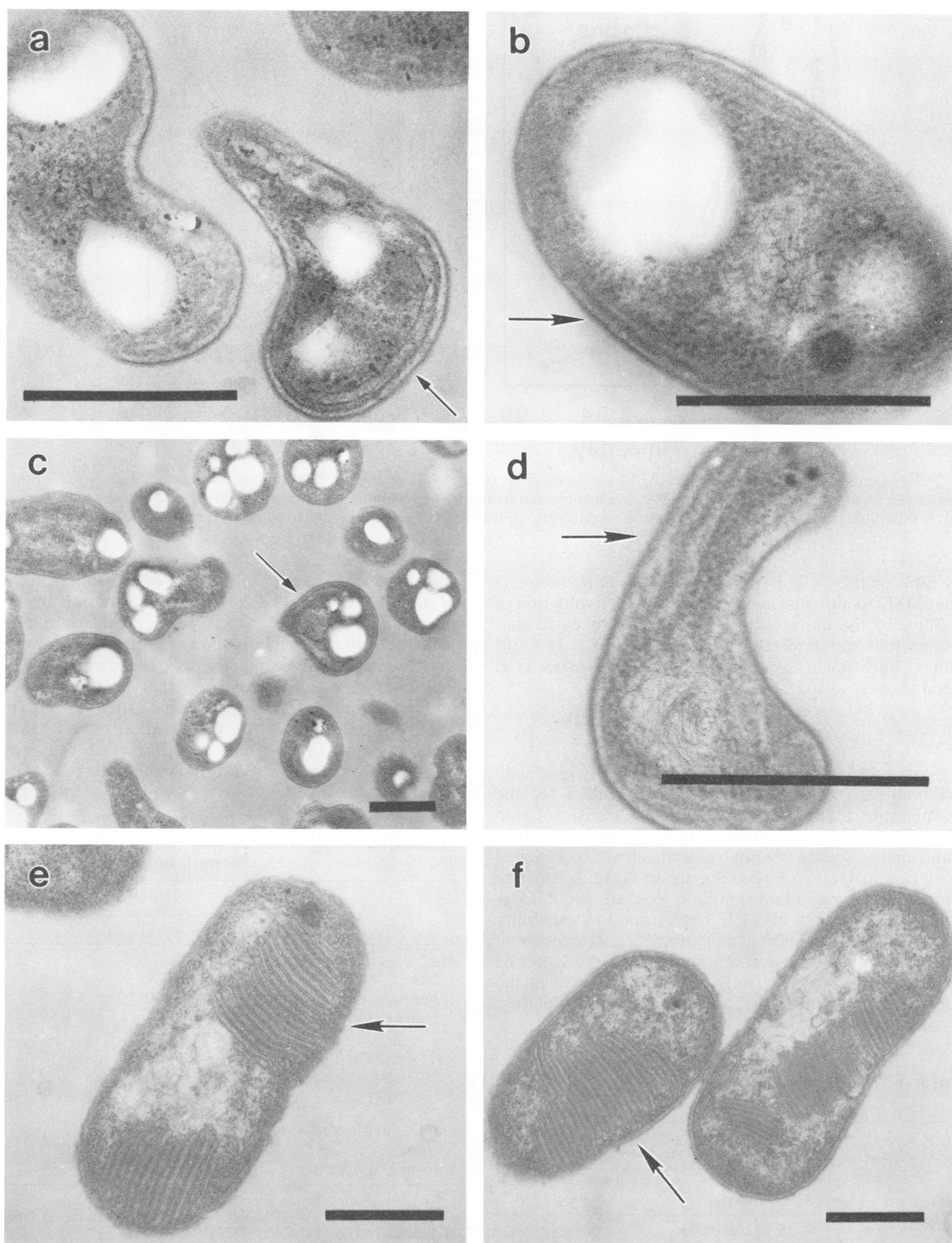
FIG. 1. Transmission electron micrographs of aquifer methanotrophs. Lipid storage granules are the electron-dense (light-colored) inclusions inside the cells. Methanotrophs in mixed cultures MM1 (A) and MM3 (B) and isolate CSC-1 (C) contain lipid inclusions when grown under the conditions used in these experiments (note type II intracytoplasmic membranes). Methanotrophs in mixed culture MM2 (D; note type II membranes) (E; note type I membranes), and *Methylomonas* sp strain MM2 (F; note type I membranes) do not. Bar, 0.5 μm.
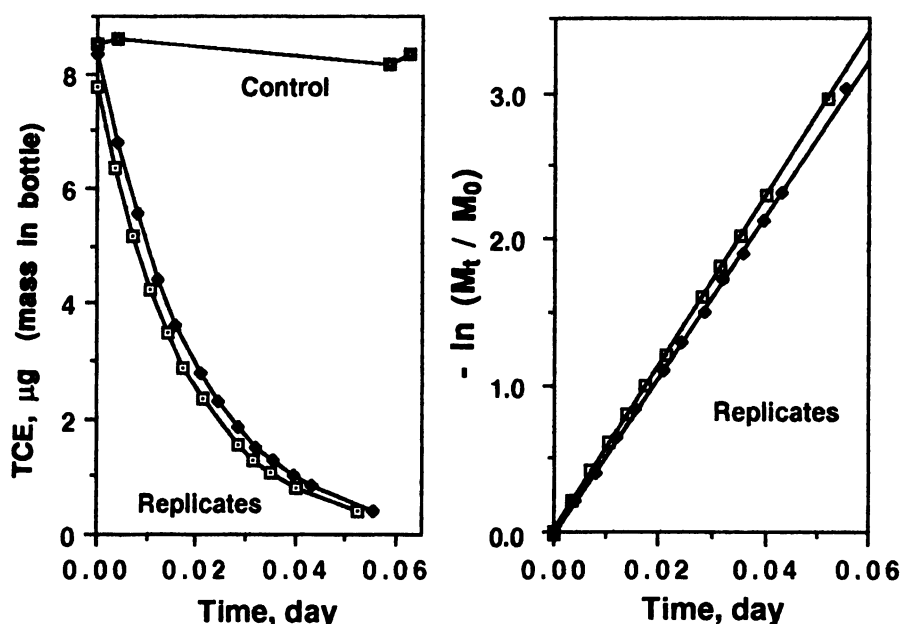
FIG. 2. Resting-cell TCE transformation by *Methylomonas* sp strain MM2 grown in Whittenbury mineral medium with 2 mM formate added as an electron donor. The data in panel A, when subjected to analysis by Monod kinetics (B), yield a pseudo-first-order rate coefficient $k'$ of 2.3 ± 0.05 liter $mg^{-1}$ $day^{-1}$. The correlation coefficients, describing the fit of the data to the rate model, are 1.00 for both replicates.

imately 80% of the TCE is transformed by *Methylomonas* sp. strain MM2 to aqueous intermediates [13].) Evaluation of the viability of the cultures used in assay 2 revealed that subcultures that had oxidized TCE contained $(2 \pm 1) \times 10^5$ CFU $ml^{-1}$, and those that had not been exposed to TCE contained $(5 \pm 2) \times 10^6$ CFU $ml^{-1}$.

## DISCUSSION

The methanotrophs used in these experiments gradually lost the ability to oxidize TCE during starvation for the growth substrate, methane. This loss of TCE transformation capability followed exponential decay. Formate, an electron donor and energy source but not a growth substrate, reduced the decay rate in *Methylomonas* sp. strain MM2, suggesting that depletion of reductant may be one of the factors contributing to the loss of TCE transformation capability during starvation. Reduction in cell numbers cannot account for the extremely low transformation rates observed after 62 h of starvation as $10^7$ CFU $ml^{-1}$ were still present in the *Methylomonas* sp. strain MM2 subcultures. Other factors,

such as deactivation of the MMO, may also account for loss of activity toward TCE. TCE transformation by the "formate" subculture of *Methylomonas* sp. strain MM2 (the subculture that was incubated with formate before the addition of TCE) was enhanced only during the first 10 h of methane starvation, suggesting that prolonged incubation with an exogenous source of electrons during methane starvation may accelerate deactivation of the MMO. Formate stimulated resting-cell transformation by *Methylomonas* sp. strain MM2, but not by mixed culture MM1 and the

TABLE 1. TCE transformation by mixed cultures starved for methane[a]

| Mixed culture | Lipid inclusions | TCE transformation rate, $k'$ = liter $mg^{-1}$ $day^{-1}$[b] | |
|---|---|---|---|
| | | No methane | 0.3 mg of methane $liter^{-1}$ |
| MM1 | Yes | 0.041 ± 0.003 | 0.046 ± 0.001 |
| MM2 | No | 0 | 0.004 ± 0.001 |
| MM3 | Yes | 0.046 ± 0.004 | 0.046 ± 0.004 |

[a] Cultures were grown in Fogel mineral medium and aerated for 15 h without methane.
[b] TCE transformation rates by cultures grown in Fogel mineral medium are lower than for those grown in Whittenbury mineral medium (14).

TABLE 2. Decay in TCE transformation rates during starvation for methane

| Subculture | Decay parameter $day^{-1}$[a] | Correlation coefficient | No. of data points |
|---|---|---|---|
| *Methylomonas* sp. strain MM2 | | | |
| None[b] | 2.3 | 0.98 | 10 |
| Formate[c] | 2.4[d] | 0.89 | 10 |
| None + formate[e] | 1.4 | 0.99 | 8 |
| Mixed culture MM1[f] | | | |
| None | 1.7 | 0.99 | 10 |
| Formate | 1.7 | 0.99 | 10 |
| Unidentified isolate CSC-1[f,g] | | | |
| None | 2.2 | 0.99 | 6 |
| None + formate | 2.1 | 0.99 | 6 |

[a] $-\ln$ (rate$_t$/rate$_0$) versus time (day).
[b] None, Subculture was incubated without formate and received no formate at the time of TCE addition.
[c] Formate, Subculture was incubated with formate, and formate was added when TCE was added.
[d] Incubation with formate increased rates only during the first 10 h (Fig. 3).
[e] None + formate, Formate was added to "None" subculture when TCE was added.
[f] Methanotrophs contain lipid inclusions.
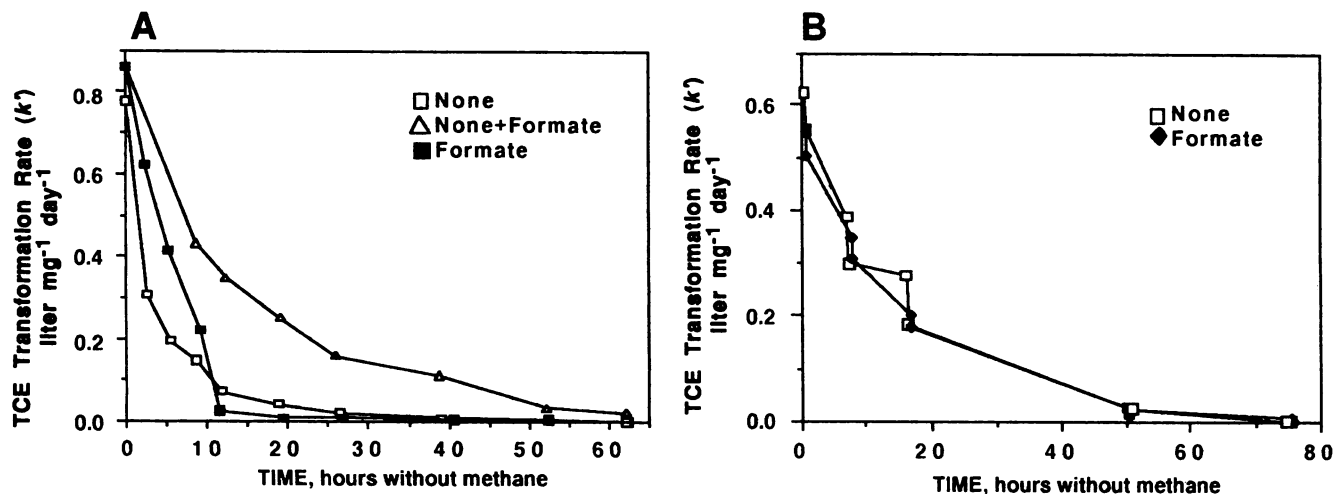[g] Unidentified isolate CSC-1 was isolated from mixed culture MM1.

FIG. 3. Effect of formate amendment on TCE transformation rates during methane starvation. The rate of TCE transformation, $k'$, is plotted against time in hours. (A) *Methylomonas* sp. strain MM2. (B) Mixed culture MM1. "None" denotes subcultures that were incubated during starvation without formate and were not amended with formate. "None + Formate" denotes subcultures that were incubated without formate, but received 2 mM formate at the same time TCE was added. "Formate" denotes subcultures that were incubated with formate and received 2 mM formate at the same time TCE was added. Note differences in scale between panels A and B.

unidentified isolate CSC-1. Since only those mixed cultures that contained lipid inclusions could still carry out resting-cell transformation of TCE after 15 h without methane, it appears that the methanotrophs that contain lipid inclusions rely on their endogenous reserves for a source of electrons and energy during starvation.
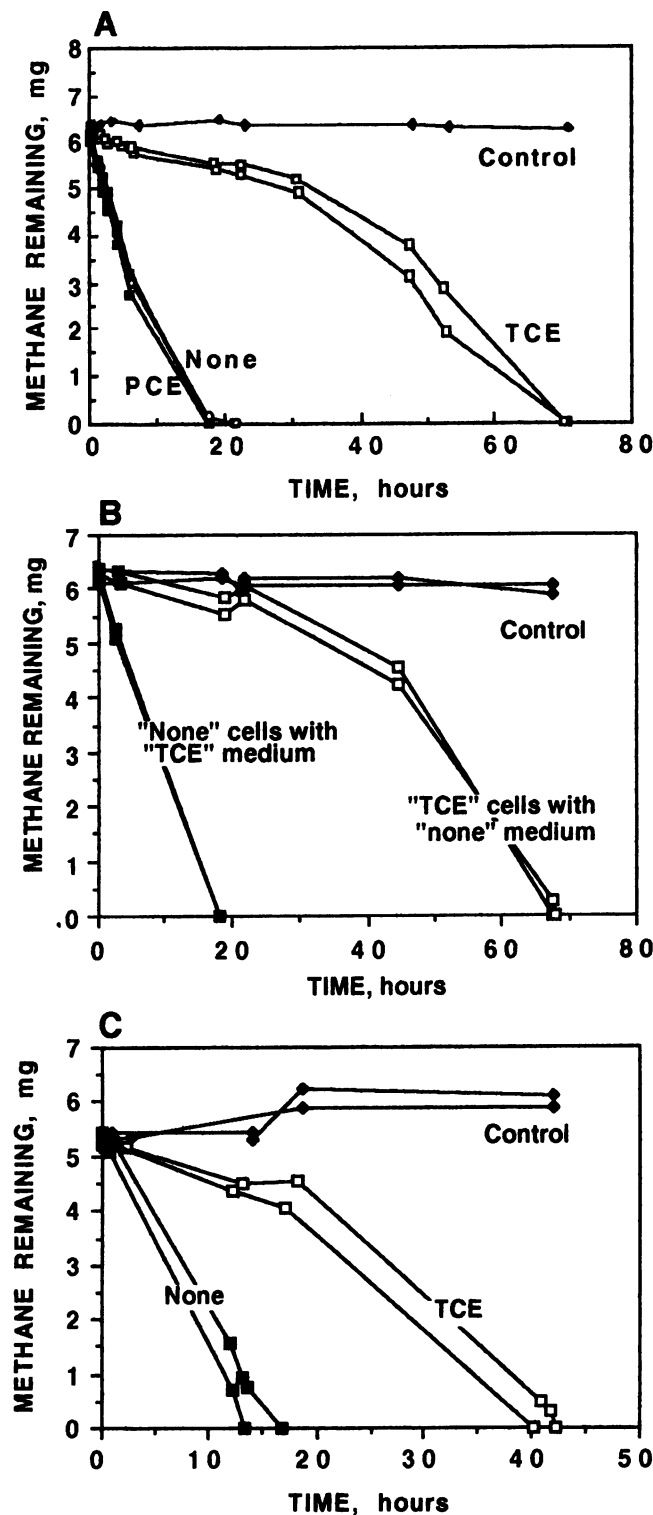
It could be argued that other differences between these methanotrophs may also contribute to the different responses to formate amendment. It has been shown that *Methylomonas* sp. strain MM2 expresses only a membrane-associated ("particulate") MMO under the growth conditions applied in these studies (12c, 14). *Methylomonas* sp. strain MM2 cell preparations did not cross-react with an immunological probe prepared against the soluble MMO of *Methylosinus trichosporium* OB3b (35a). Mixed culture MM1 and SH-1, an unidentified type II methanotroph isolated from mixed culture MM1, did cross-react with the soluble MMO probe (35a, 36), indicating that methanotrophs in mixed culture MM1 expressed soluble MMO similar to that of *Methylosinus trichosporium* OB3b under the growth conditions applied in these studies. However, whereas formate did not stimulate resting-cell TCE transformation in mixed culture MM1 and isolate CSC-1, it did stimulate resting-cell TCE transformation in *Methylosinus trichosporium* OB3b (30). Researchers studying TCE transformation by *Methylosinus trichosporium* OB3b do not report the accumulation of storage granules by this organism (30, 37). This supports the hypothesis that the presence of lipid inclusions, and not the differences in the MMOs expressed by these different methanotrophs, may be responsible for the lack of stimulation of TCE transformation by formate in mixed culture MM1 and isolate CSC-1.

Our experiments suggest that the toxic effect of TCE on microbial cells may be based on the oxidation of TCE, and not on the presence of TCE itself. TCE oxidation resulted in significant reduction of subsequent methane oxidation capability and reduced the number of viable cells by more than an order of magnitude. The concentration of TCE used in these experiments, 6 mg liter$^{-1}$, unlike TCE concentrations in the 50-mg liter$^{-1}$ range (13), did not inhibit concurrent TCE

transformation and methane utilization. Furthermore, cells preexposed to PCE, a solvent chemically similar to TCE but which is not transformed, did not exhibit a reduced ability to utilize methane. This supports the suggestion that TCE itself is not the toxic agent; neither were the intermediates that remain in solution after TCE has been transformed by *Methylomonas* sp. strain MM2. Carbon monoxide, a transient intermediate in the transformation of TCE by *Methylomonas* sp. strain MM2 (12b), is a competitive inhibitor of TCE oxidation by *Methylomonas* sp. strain MM2, but is not otherwise toxic (unpublished data).

Studies of mammalian liver enzymes have shown that TCE must be metabolically activated to exert toxicity towards cells. The activation reaction is an oxygenation that leads to the formation of reactive intermediates, including the TCE epoxide, chloral (6, 15, 23, 24), and metabolites that bind irreversibly to macromolecules within the cell, including protein, DNA, and RNA (4, 5, 24). Wackett and Householder (43) demonstrated that TCE had to be metabolically activated by the toluene dioxygenase in *P. putida* F1 to exert a toxic effect. They hypothesized that the toxicity resulting from TCE oxidation was based on the modification of intracellular molecules by reactive intermediates (43). The TCE-epoxide has been proposed to be the resultant product of oxygenation of TCE by the MMO (12d, 13, 21, 30). Dichloroethylene epoxide was detected during the in situ biodegradation study conducted at Moffett Field (34, 35) and during dichloroethylene transformation by methanotrophic consortia (18, 19a). It is plausible that the toxic effect observed in our methanotrophs is the result of the TCE-epoxide, or another highly reactive intermediate, damaging intracellular molecules.

TCE oxidation toxicity is an important consideration in the methanotrophic bioremediation of TCE. Even at low TCE concentrations, TCE transformation could result in a significant decay of the active biomass, which could be expected to increase with increasing amounts of TCE transformed. In the development and modeling of process applications, the decay in active biomass due to TCE oxidation should be evaluated. Different methanotrophs, and indeed

FIG. 4. Effect of TCE oxidation on subsequent methane utilization by *Methylomonas* sp. strain MM2 and mixed culture MM1. Total mass of methane, milligrams, remaining in the bottles is plotted against time in hours. "None" denotes culture incubated without TCE; "TCE" denotes culture incubated with 6 mg of TCE liter$^{-1}$; "PCE" denotes culture incubated with PCE. (A) *Methylomonas* sp. strain MM2 assay 1. PCE was not transformed by the subcultures. Ratio of TCE oxidized per cell dry weight was 0.028. (B) *Methylomonas* sp. strain MM2 assay 2. TCE oxidized per cell dry weight was 0.017. Culture medium from the "TCE" subcultures was switched with that from the "None" subcultures. (C) Mixed culture MM1. TCE oxidized per cell dry weight was 0.016.

thermore, formate addition during in situ aquifer remediation will increase the expense and could potentially stimulate growth of nonmethanotrophic biomass that would compete for nutrients and oxygen. The effect of formate addition should be evaluated with the methanotrophs specific to the treatment application before formate addition is considered. Alternatively, microorganisms that accumulate storage granules such as lipid inclusions may be of value in treatment applications because of the endogenous reserves of reducing power and energy.

## ACKNOWLEDGMENTS

## REFERENCES

1. **Alvarez-Cohen, L., and P. L. McCarty.** 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57:**228–235.
2. **Arciero, D., T. Vannelli, M. Logan, and A. B. Hooper.** 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochem. Biophys. Res. Commun. **159:**640–643.
3. **Asenjo, J. A., and J. S. Suk.** 1986. Microbial conversion of methane into poly-β-hydroxybutyrate (PHB): growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. **64:**271–278.
4. **Bergman, K.** 1983. Interactions of trichloroethylene with DNA in vitro and with RNA and DNA of various mouse tissues in vivo. Arch. Toxicol. **54:**181–193.
5. **Bolt, H. M., and J. G. Filser.** 1977. Irreversible binding of chlorinated ethylenes to macromolecules. Environ. Health Perspect. **21:**107–112.
6. **Bonse, G., and D. Henschler.** 1976. Chemical reactivity, biotransformation, and toxicity of polychlorinated aliphatic compounds. Crit. Rev. Toxicol. **4:**395–409.
7. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformation of 1- and 2-carbon halogenated aliphatic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45:**1286–1294.
8. **Davies, S. L., and R. Whittenbury.** 1970. Fine structure of methane and other hydrocarbon-utilizing bacteria. J. Gen. Microbiol. **61:**227–232.
9. **Fogel, M. M., A. R. Taddeo, and S. Fogel.** 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. **51:**720–724.
10. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol

the different classes of bacteria shown to oxidize TCE, may be affected by TCE oxidation toxicity to different degrees. This possibility represents a valuable area for further research.

Stimulation of resting-cell TCE transformation by formate addition may not be universal among methanotrophs. Fur-

and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56**:1279–1285.

11. **Ghiorse, W. C., and D. L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. **24**:213–224.

12. **Harker, A. R., and Y. Kim.** 1990. Trichloroethylene degradation by two independent aromatic-degrading pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. **56**:1179–1181.

12a.**Henry, S. M.** 1989. Abstr. Tech. Papers Annu. Water Pollution Control Federation Conf. 1989, p. 27.

12b.**Henry, S. M., C. S. Criddle, and D. Grbić-Galić.** 1989. Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem. 1989, abstr. 41, p. 70.

12c.**Henry, S. M., A. A. DiSpirito, M. E. Lindstrom, and D. Grbić-Galić.** 1989. Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K69, p. 256.

12d.**Henry, S. M., and D. Grbić-Galić.** 1986. Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, Q64, p. 294.

13. **Henry, S. M., and D. Grbić-Galić.** 1989. TCE transformation by mixed and pure ground water cultures, p. 109–125. *In* P. V. Roberts, L. Semprini, G. D. Hopkins, D. Grbić-Galić, P. L. McCarty, and M. Reinhard (ed.), *In-situ* aquifer restoration of chlorinated aliphatics by methanotrophic bacteria. EPA Technical Report EPA/600/2-89/033. R. S. Kerr Environmental Research Laboratory, U.S. EPA, Ada, Okla.

13a.**Henry, S. M., and D. Grbić-Galić.** 1989. Abstr. Int. Symp. Processes Governing the Movement and Fate of Contaminants in the Environment 1989, p. A4.

14. **Henry, S. M., and D. Grbić-Galić.** 1990. Effect of mineral media on trichloroethylene oxidation by aquifer methanotrophs. Microb. Ecol. **20**:151–169.

14a.**Henry, S. M., F. Thomas, and D. Grbić-Galić.** 1988. Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem. 1988, abstr. 2261, p. 74.

15. **Henschler, D.** 1985. Halogenated alkenes and alkynes, p. 317–347. *In* M. W. Anders (ed.), Bioactivation of foreign compounds. Academic Press, Inc., Orlando, Fla.

16. **Henson, J. M., M. V. Yates, and J. W. Cochran.** 1989. Metabolism of chlorinated methanes, ethanes and ethylenes by a mixed bacteria culture growing on methane. Ind. Microbiol. **4**:29–35.

17. **Hou, C. T.** 1984. Microbiology and biochemistry of methylotrophic bacteria, p. 1–53. *In* C. T. Hou (ed.), Methylotrophs: microbiology, biochemistry, and genetics. CRC Press, Inc., Boca Raton, Fla.

18. **Janssen, D. B., G. Grobben, and B. Witholt.** 1987. Toxicity of chlorinated aliphatic hydrocarbons and degradation by methanotrophic consortia. Proc. 4th Eur. Congr. Biotechnol. **3**:515–518.

19. **Lanzarone, N. A., K. P. Mayer, M. E. Dolan, D. Grbić-Galić, and P. L. McCarty.** 1989. Batch exchange soil column studies of biotransformation by methanotrophic bacteria, p. 126–146. *In* P. V. Roberts, L. Semprini, G. D. Hopkins, D. Grbić-Galić, P. L. McCarty, and M. Reinhard (ed.), *In-situ* aquifer restoration of chlorinated aliphatics by methanotrophic bacteria. EPA Technical Report EPA/600/2-89/033. R. S. Kerr Environmental Research Laboratory, U.S. EPA, Ada, Okla.

19a.**Leahy, M. C., M. Dooley-Danna, M. S. Young, and M. S. Fogel.** 1987. Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K178, p. 232.

19b.**Leahy, M. C., M. Findlay, and S. Fogel.** 1989. Proc. 2nd Natl. Conf. Hazardous Materials Control Res. Inst. Biotreatment, p. 3–9.

20. **Linton, J. D., and R. E. Cripps.** 1978. The occurrence and identification of intracellular polyglucose storage granules in *Methylococcus* NCIB 11083 grown in chemostat on methane. Arch. Microbiol. **117**:41–48.

21. **Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R. L. Tyndall, and P. J. Gilmer.** 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. **54**:951–956.

22. **Maltoni, C., and G. Lefemine.** 1974. Carcinogenicity bioassays of vinylchloride. I. Research plan and early results. Environ. Res. **7**:387–396.

23. **Miller, R. E., and F. P. Guengerich.** 1982. Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry **21**:1090–1097.

24. **Miller, R. E., and F. P. Guengerich.** 1983. Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. Cancer Res. **43**:1145–1152.

25. **Monod, J.** 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. **3**:371–394.

26. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**:949–954.

27. **Nelson, M. J. K., S. O. Montgomery, E. J. O'Neill, and P. H. Pritchard.** 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. **52**:383–384.

28. **Nelson, M. J. K., S. O. Montgomery, and P. H. Pritchard.** 1988. Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54**:604–606.

29. **Norris, J. R., and H. Swain.** 1971. Staining bacteria, p. 105–133. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5A. Academic Press, Inc., (London), Ltd., London.

30. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55**:2819–2826.

31. **Parsons, F., P. R. Wood, and J. DeMarco.** 1984. Transformation of tetrachloroethene and trichloroethene in microcosms and ground water. J. Am. Water Works Assoc. **76**:56–59.

32. **Poindexter, J. A.** 1981. Oligotrophy: fast and famine existence. Adv. Microb. Ecol. **5**:63–89.

33. **Rannug, U., A. Johansson, C. Ramel, and C. A. Wachtmeister.** 1974. The mutagenicity of vinyl chloride after metabolic activation. Ambio **3**:194–197.

34. **Semprini, L., G. Hopkins, and P. Roberts.** 1989. Results of biostimulation and biotransformation experiments, p. 65–90. *In* P. V. Roberts, L. Semprini, G. D. Hopkins, D. Grbić-Galić, P. L. McCarty, and M. Reinhard (ed.), *In-situ* aquifer restoration of chlorinated aliphatics by methanotrophic bacteria. EPA Technical Report EPA/600/2-89/033. R. S. Kerr Environmental Research Laboratory, U.S. EPA, Ada, Okla.

35. **Semprini, L., P. V. Roberts, G. D. Hopkins, and P. L. McCarty.** 1990. A field evaluation of *in situ* biodegradation of chlorinated ethenes, Part 2. Results of biostimulation and biotransformation experiments. Groundwater **28**:715–727.

35a.**Tsien, H. C.** Personal communication.

36. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. **56**:2858–2865.

37. **Tsien, H. C., G. A. Brusseau, R. S. Hanson, and L. P. Wackett.** 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55**:3155–3161.

38. **Vannelli, T., M. Logan, D. M. Arciero, and A. B. Hooper.** 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56**:1169–1171.

39. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722–736.

40. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080–1083.

41. **Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson.** 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55**:2960–2964.

42. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichlo-

roethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.

43. **Wackett, L. P., and S. R. Householder.** 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. **55:**2723–2725.

44. **Westrick, J. J., J. W. Mello, and R. F. Thomas.** 1984. The ground water supply survey. J. Am. Water Works Assoc. **76:**52–59.

45. **Whittenbury, R., K. C. Phillips, and J. F. Wilkinson.** 1970. Enrichment, isolation, and some properties of methane-utilizing bacteria. J. Gen. Microbiol. **24:**225–233.

46. **Wilson, J. T., and B. H. Wilson.** 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. **29:**242–243.